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Transition Metal-Binding Proteins from Three Chesapeake Bay Fish Species

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Three species of Chesapeake Bay fish were collected, and endogenous levels of metal binding protein (MBP) were determined. In addition, the induction of metal-binding proteins by cadmium was studied. Livers from freshly caught fish were extracted and chromatographed on Sephadex G-75 to resolve MBP in the 5 to 20 kdalton range. All species studied exhibit measurable but varied levels of endogenous MBPs in the molecular weight range investigated, mostly as a copper protein complex. Upon induction with cadmium, the total MBP content increased in both catfish (*Ictalurus punctatus*) and striped bass (*Morone saxatilis*), with significant amounts of cadmium bound to the protein. In white perch (*Morone americana*), induction of MBPs with cadmium could not be demonstrated due to the large amount of constitutive Cu-BP present, although significant quantities of cadmium were bound to MBP. Electrophoresis in polyacrylamide gel was used to further identify these MBPs. Electrochemical analysis of the MBPs by polarography indicated that the wave properties of the fish MBPs resemble that of rat metallothionein. In conclusion, these studies indicate that: MBPs are present in estuarine fish from the Chesapeake Bay; concentrations of MBPs and their inducibility by exogenous cadmium vary with species, and fish MBPs may be related to mammalian metallothionein.

Introduction

Striped bass (*Morone saxatilis*) and white perch (*Morone americana*) are two species of fish coinhabiting in the Chesapeake Bay area. Considered the noblest of Bay creatures, striped bass have been a sporting as well as gourmet favorite of many. However, striped bass spawning population has undergone an alarming reduction in size in the last few years. The annual index of its reproductive success has reached an all time low approaching oblivion. A legislative moratorium on fishing for this species along its migratory route of the Atlantic coast and in the Chesapeake Bay has now been imposed. White perch, on the other hand, appears to thrive well. It is thus important to learn what are the causes for the decline in striped bass population. What selective differences are there between the two *Morone* species which account for the decline in *M. saxatilis* and the apparent stability of *M. americana*?

Several factors have been attributed to the peril of striped bass; among them acid rain, overfishing, and land-leached contaminants, such as heavy metals. This study is to examine whether striped bass and other fish species in the same natural environment possess different defense mechanisms against toxicity of metals, particularly transition IB and IIB metals, copper, zinc, and cadmium. In mammals, the inducible metal-binding protein metallothionein has been suggested to have a detoxification function (*1*). Results to be presented in this report will show that similar, but not identical, metal-binding protein(s) exist in these and other fish species examined. Their constitutive levels and inducibility, however, differ, with striped bass showing only about 10% as much of this protein as white perch.

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Materials and Methods

Fish

Fish species used in this study were caught and identified on board the Ridgely Warfield, a research vessel of the Chesapeake Bay Institute, Johns Hopkins University, in April 1984, under the cruise-directorship of Dr. Robert Chapman.

Isolation of Cystolic Metal-Binding Proteins by Gel-Permeation Chromatography on Sephadex G-75

Fresh fish livers were homogenized in 0.1 M ammonium formate, pH 7.4 (1:4, *w/v*), heat-treated at 80°C for 2 min and centrifuged at 40,000*g* for 30 min. Radioactive ^{109}Cd

(40,000 cpm/mL) was added to the supernatant prior to chromatography. An aliquot of supernatant (6–10 mL) was applied to a Sephadex G-75 column (2.5 × 72.5 cm) and eluted with 0.05 M Tris HCl (pH 7.4) and 5 mM β-mercaptoethanol at a flow rate of 60 mL/hr. Fractions (4 mL) were collected and analyzed for UV absorption at 254 nm, stable Cd, Zn, and Cu, and radioactive ^{109}Cd. Recovered ^{109}Cd was detected by a Beckman Gamma 4000 counter. Stable Cd as well as Zn and Cu were measured by atomic absorption spectrophotometry. The pre-

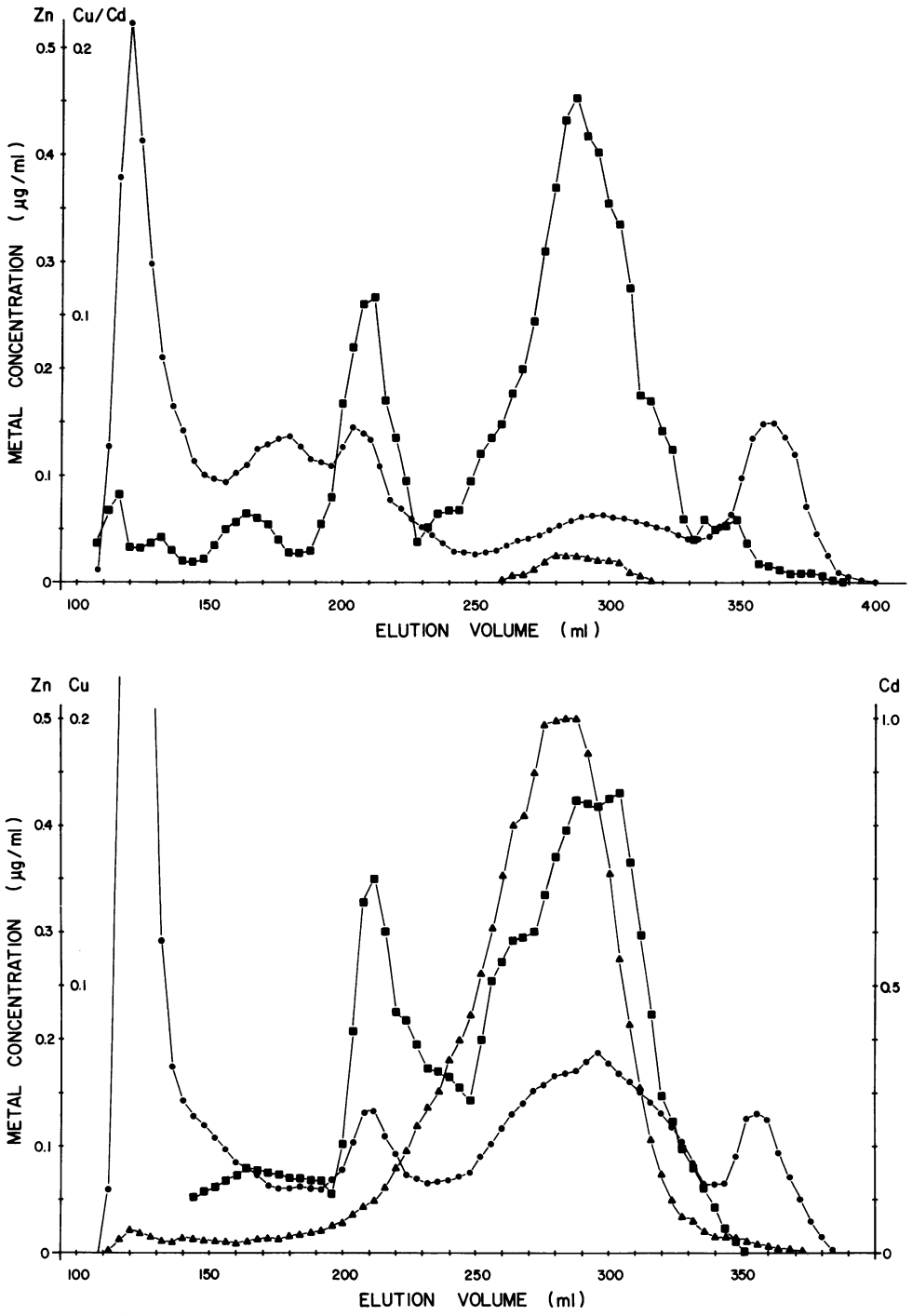

FIGURE 1. Isolation of cytosolic metal-binding proteins by gel-permeation chromatography on Sephadex G-75: (▲) Cd; (■) Cu; (●) Zn. Liver homogenates were chromatographed on a Sephadex G-75 column (2.5 × 72.5 cm) and eluted with 0.05 M Tris HCl, pH 7.4, a flow rate of 60 mL/hr. (See text for determination.)In Cd induction studies, $CdCl_2$ was injected intramuscularly in doses of 0.5, 1.0, and 2.0 mg/kg over a 3-day period. Fish were sacrificed 24 hr after the last injection: Proteins from (*A*) striped bass, constitutive; (*B*) striped bass, induced.

sented data have been normalized to equal volumes of cytosol applied to the column.

In Cd-induction studies, cold $CdCl_2$ was injected intramuscularly in doses of 0.5, 1.0, and 2.0 mg/kg over a 3-day period. Fish were sacrificed 24 hr after the last injection.

Resolution of Cd-Binding Protein by DEAE-Sephadex A-50 Anion Exchange Chromatography

The major Cd binding peak from Sephadex G-75 chromatography of induced striped bass cytosol was rechro-

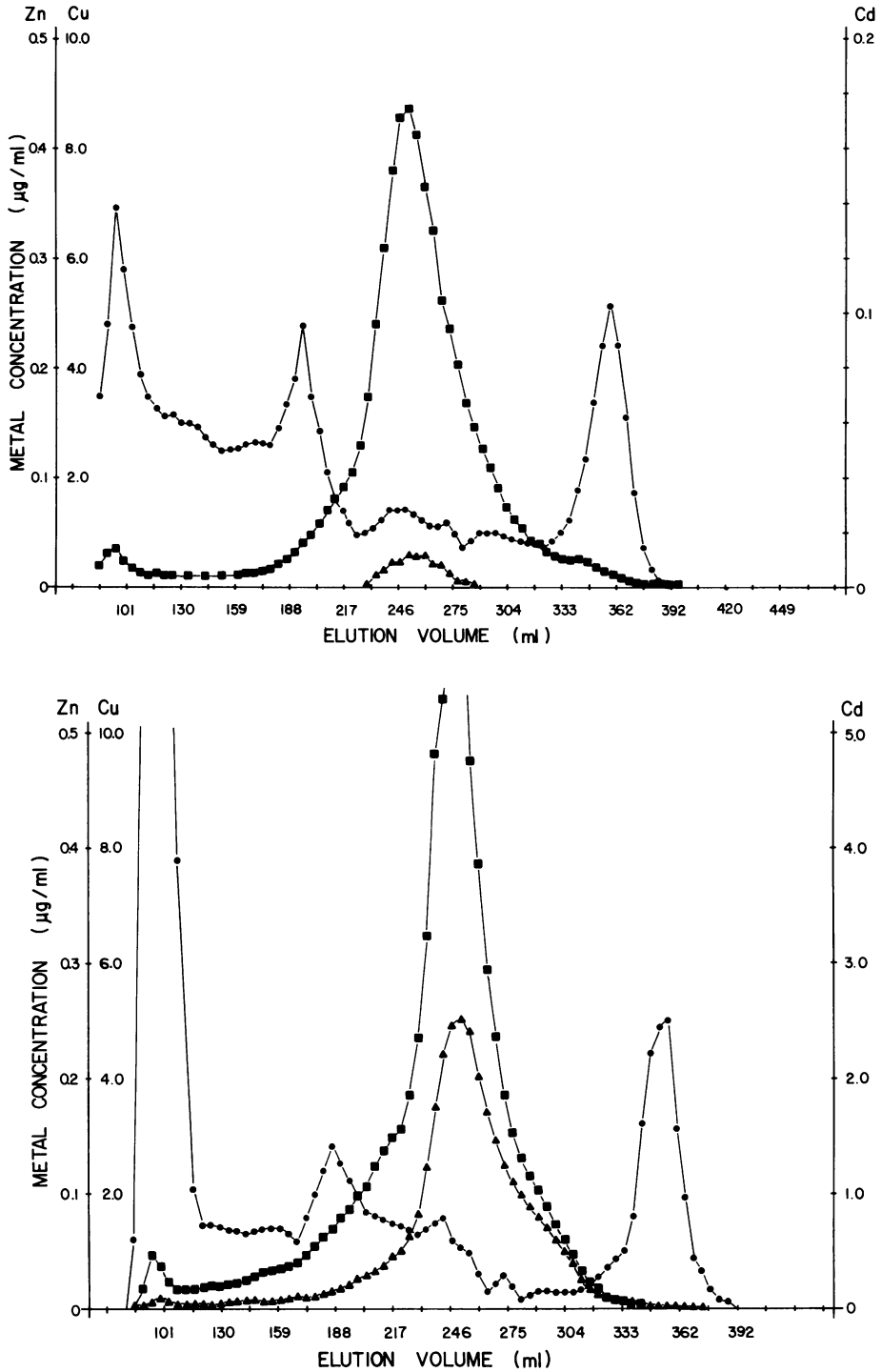

FIGURE 2. Isolation of cytosolic metal-binding proteins by gel-permeation chromatography on Sephadex G-75: (▲) Cd; (■) Cu; (●) Zn. Protein from (*A*) white perch, constitutive; (*B*) white perch, induced. Details as in Fig. 1.

matographed on a DEAE–A-50 Sephadex column (1.5 × 25 cm). The column was equilibrated and washed following sample application with 0.05 M Tris HCl buffer, pH 8.5, at room temperature. The column was eluted with a linear gradient from 0.05 to 0.5 M Tris-HCl. Fractions of 4 mL were collected and analyzed for Cd, Zn, and Cu by atomic absorption spectrometry.

Determination of Metal Content

Metal concentration (Cd, Zu, and Cu) in chromatographic fractions were determined by atomic absorption spectrophotometry on a Varian (Model AA5) spectrophotometer. Samples were aspirated directly without dilution.

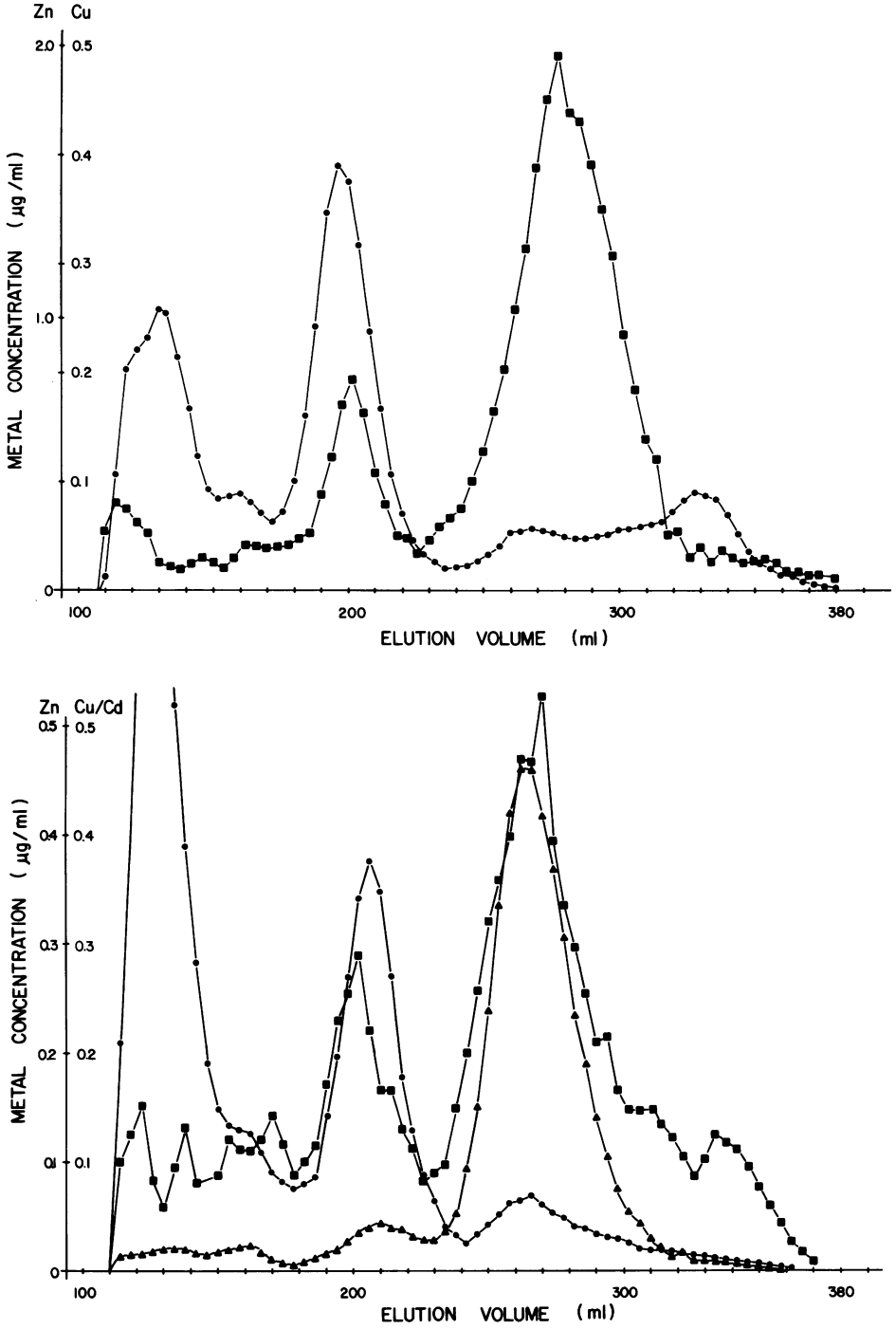

FIGURE 3. Isolation of cytosolic metal-binding proteins by gel-permeation chromatography on Sephadex G-75: (▲) Cd; (■) Cu; (●) Zn. Protein from (*A*) catfish, constitutive; (*B*) catfish, induced. Details as in Fig. 1.

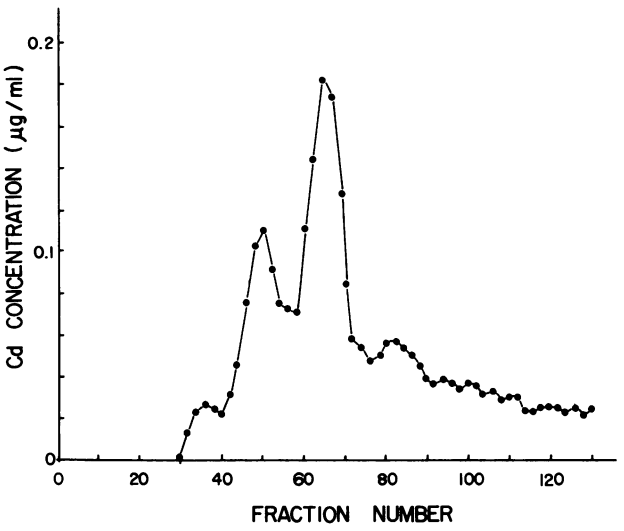

FIGURE 4. Resolution of striped bass Cd-binding protein by DEAE-anion-exchange chromatography. The major Cd-binding fractions of induced striped bass cytosol from Sephadex G-75 chromatography were pooled and rechromatographed on a DEAE A-50 column (1.5 × 25 cm), with a linear gradient from 0.05 to 0.5 M Tris. Fractions (4 mL) were collected and analyzed for stable Cd by atomic absorption spectrophotometry.

Differential Pulse Polarography of Heat-Treated Fish Hepatic Cytosol

Differential pulse polarography by the Brdicka procedure was carried out according to Palecek and Pechan (*2*) and Olafson and Sim (*3*) by use of a Metrohm E502 analyzer. Analysis was performed in 10 mL aliquots of supporting electrolyte by scanning from −1.30 to −1.60 V at −5 mV/sec. The mercury drop time was 0.5 sec and the sensitivity setting 20 μA/mm. The Brdicka cobalt electrolyte was used without surface-active agent. The electrolyte was purged with high purity nitrogen for 8 min prior to addition of sample and then for an additional 2 min.

Polyacrylamide Gels of Fish Hepatic Cytosols

Fish heat-treated cytosol samples (5–10 μL) were run on a polyacrylamide gel electrophoresis system consisting of 5% stacking gel and a 7.5% to 17% gradient gel using a Sturdier vertical slab gel unit (model SE400, Hoefer Scientific Instruments) and a Buchler 3-1500 constant power supply. The chemical polymerization of the polyacrylamide gel was performed by using ammonium persulfate and TEMED as catalyst–redox system. A small amount of glycerol was added to minimize turbulence during gradient pouring. Both stacking and gradient gel contained 0.1% SDS. Before electrophoresis all samples were diluted 1:1 with a denaturing solution containing 4% SDS and 10% β-mercaptoethanol and heated at 80°C for 2 min to minimize negative charge differences and disrupt disulfide linkages. The running buffer for the electrophoresis also contained 0.1% SDS.

Purified rat liver metallothionein standard used as a marker protein was prepared according to Ohi et al. (*4*).

Results

Inspection of the Sephadex G-75 profiles for all three species of fish (Figs. 1–3) indicates that low molecular weight metal-binding proteins (MBP) are present in fish

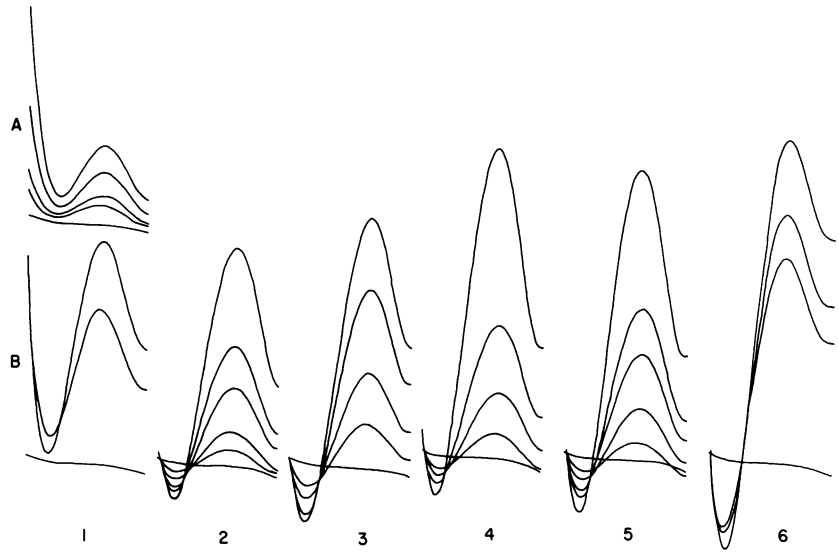

FIGURE 5. Differential pulse polarography was performed using a Metrohm E 502 analyzer on 10 mL aliquots of supporting electrolyte by scanning from −1.30 to −1.60 V at −5 mV/sec. (See text for details.) From left to right: with multiple scanning with increasing sample volumes as shown in parentheses: (1) (*A*) purified rat liver metallothionein, standard 100 μg/mL (50, 100, 200, 400 μL in ascending order) and (*B* heat-treated rat liver cytosol, Cd-induced (100, 200 μL); (2) striped bass liver cytosol (5, 10, 20, 30, 70 μL); (3) striped bass liver cytosol, Cd-induced (5, 10, 20, 30 μL); (4) catfish liver cytosol, (5, 10, 20, 60, μL); (5) catfish liver cytosol, Cd-induced (5, 10, 20, 30, 70 μL); (6) white perch liver cytosol (10, 15, 20 μL). Abscissa: in volts from −1.30 to −1.60; ordinate: nA in arbitrary units.

obtained from the natural environment. In all three fish species, the naturally occurring MBPs are predominantly copper complexes, with lesser amounts of zinc and only traces of cadmium present. The ^{109}Cd-binding data demonstrate that naturally occurring MBPs will bind cadmium with a high affinity, relative to other intracellular ligands, in spite of their significant copper loads. The relative elution volumes (K_a) for these MBPs indicate that striped bass (K_a = 0.71) and catfish (K_a = 0.69) have similar apparent molecular weights, while the white perch BP behaves on the Sephadex G-75 column under these experimental conditions as a larger protein (K_a = 0.58).

Injections of cadmium intramuscularly results in significant quantities of cadmium binding to the MBPs. In the case of the catfish and striped bass, the total MBP content increased following cadmium induction. With the white perch it is not possible to draw a similar conclusion due to the extremely high levels of copper-BP present in the uninduced fish.

Only in the case of the striped bass was DEAE–anion-exchange chromatography successful (Fig. 4). Insufficient quantities of catfish MBP were available, while white perch gave low quality profiles (smeared peaks) possibly due to the presence of large quantities of copper. For the striped bass, the DEAE results indicated two major cadmium-containing peaks. The possibility of additional minor cadmium-containing peaks cannot be ruled out.

Analysis of the crude MBP preparations obtained from Sephadex G-75 chromatography by differential pulse polarography indicated that the MBPs from all three fish species exhibited characteristics similar to mammalian metallothioneins (Fig. 5). This conclusion is based on the close correspondence of the electrode voltage at the reductive wave peak between rat MT and the fish MBPs (at −1.45 V). When the polarographic response is plotted versus increasing additions of cellular proteins to the reaction vessel (Fig. 6) there is evidence for induction of BPs with cadmium injections for catfish and striped bass. Furthermore, it is apparent that either the white perch

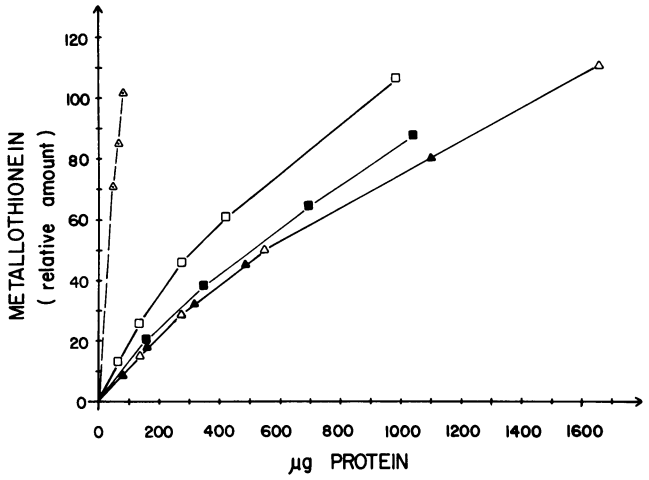

FIGURE 6. Comparison of polarographic responses for normal and induced fish. The data in Fig. 5 were replotted to show polarographic response versus amount of added cytosolic protein: (△) naive white perch; (■) striped bass induced; (▲) striped bass control; (□) catfish induced; (△) catfish control.

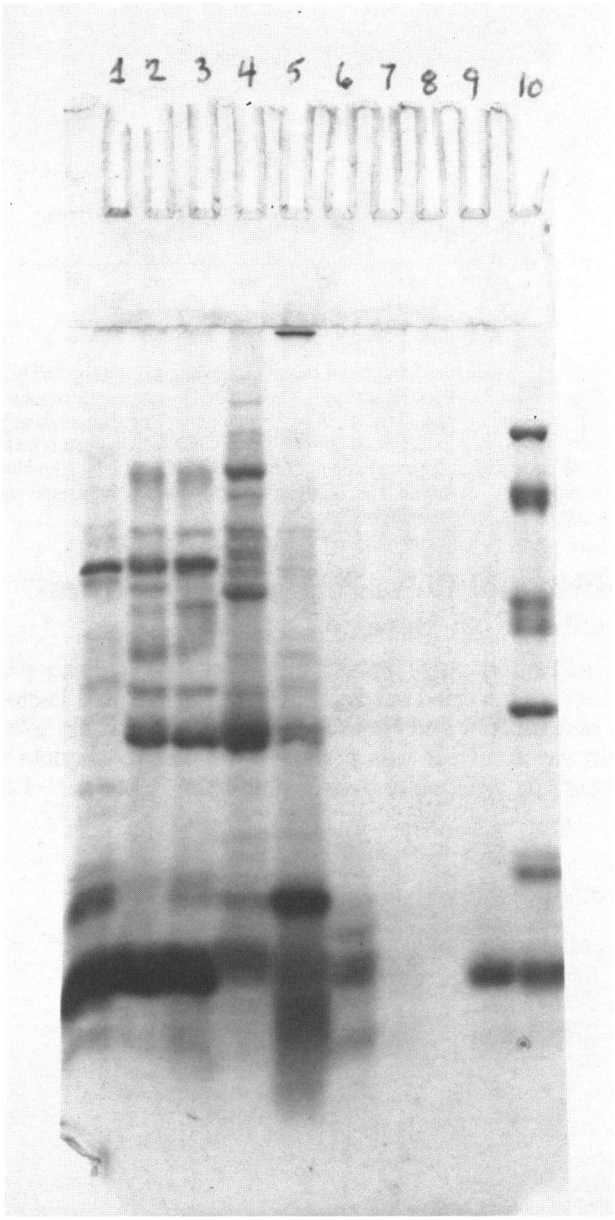

FIGURE 7. Polyacrylamide gel electrophoresis of heat-treated fish hepatic cytosols. Polyacrylamide gradient gel (7.5–17%) was used to analyze heat-treated fish hepatic cytosols. Gels were stained with Coomassie Brilliant Blue stain in lanes from left to right: (1) Cd-induced rat liver, heat-treated cytosol; (2) catfish liver, control cytosol; (3) catfish liver, Cd-induced cytosol; (4) striped bass liver, control cytosol; (5) striped bass liver, Cd-induced cytosol; (6) white perch liver, control cytosol; (7) striped bass, ion-exchange, peak A; (8) striped bass, ion-exchange, peak B; (9) purified rat liver, MT standard; (10) protein markers (phosphorylase B, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 20,100; α-lactalbumin, 14,400).

Table 1. Metallothionein in fish and other marine vertebrates.

Species	Tissue	Associated metals	Apparent MW	Reference
Carassius auratus L. (gold fish)	Liver			(*5*)
Sebastodes caurinus (copper rock fish)	Liver		11,000	(*6*)
Halichoerus grypus (Atlantic grey seal)			9,000	(*6*)
Anguilla anguilla (eel)	Liver, gill	Cd, Zn, Cu		(*7*)
Pleuronectes plastessa (plaice)	Liver		14,000; 13,000; 15,000	(*8,9*)
Leptocottus armatus (Pacific staghorn sculpin)				(*10*)
Cyrinus carpio (carp)	Liver	Cd, Cu, Zn	12,400	(*11*)
Oncorhynchus kisutch (coho salmon)	Liver, gill, kidney	Cu		(*12,13*)
Salmo gairdneri (rainbow trout)			15,000	(*14–19*)
Perca fluviatilis (perch)				
Morone saxitalis	Liver	Cd, Cu, Zn		This study
Morone americana	Liver	Cd, Cu, Zn		This study
Salmo trutta tarioli (brown trout)				(*14*)
Rutilus rutilus (roach)				
Fundulus heteroclitus (killifish)				(*20*)

BP has a significantly greater response (μamps per μmole protein) or there are much greater quantities of the MBP naturally present in white perch. The latter conclusion is supported by the metal content of the MBP peak in Sephadex G-75 profiles (Figs. 1*A*, 2*A*, 3*A*).

The polyacrylamide gradient gel electrophoresis results are given in Figure 7. Coomassie Brilliant Blue staining indicates that all three fish species, normal as well as cadmium induced, exhibit a band which migrates with the same characteristics as authentic rat MT-I. Significant quantities of this protein are present in normal catfish. Of particular interest is the strongly staining low molecular weight band in the induced striped bass lane. This protein may represent cadmium-induced induction of a new cellular protein. Further studies will be necessary to investigate this possibility.

Discussion

Several species of fish have been previously examined for the presence of metallothionein-related metal-binding proteins in the liver. These results are summarized in Table 1. As shown in the table, proteins with apparent molecular weight from 9 to 15.3 kdalton exists in these fish. Except in plaice, where one of the proteins has been shown to share a conserved mammalian type of metallothionein sequence, the primary structure and function of most metal-binding proteins are yet unclear.

A major finding in this study is the drastic difference between the amount of copper and metal-binding protein in two related species of the same genus of fish. White perch accumulates between 100 and 1000 times more copper in the liver than striped bass. It is thus significant to observe that the corresponding binding protein is at least 10-fold higher in the former.

Several mechanisms can explain this species variation, two of which are particularly attractive: (a) differential affinity for metals due to alteration in the intracellular binding proteins and (b) similar proteins but differential expression due to amplified gene copies. These are testable explanations and are indeed being examined experimentally.

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